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ORIGINAL ARTICLE

EPIDERMAL GROWTH FACTOR RESTORES NORMAL LEVELS OF MYOSIN EXPRESSION IN SUBMANDIBULAR SALIVARY GLANDS OF RATS TREATED WITH BOTULINUM TOXIN

Mohamed Shamel[1], Mahmoud M Al Ankily[2], Mahmoud M Bakr[3]

[1] Lecturer, Oral Biology Department, Faculty of Dentistry, October University of Modern Sciences and Arts, Cairo, Egypt,
[2] Lecturer, Oral Biology Department, Faculty of Dentistry, The British University in Egypt, Cairo, Egypt,
[3] Lecturer, General Dental Practice, School of Dentistry and Oral Health, Griffith University, Queensland, 4222, Australia.

ABSTRACT:
Botulinum toxin (BTX) has a number of cosmetic, medical and dental applications. One of the dental applications of BTX is treatment of excessive salivation. Local BTX injection into salivary glands has an effect on the histology, and integrity of the tissues. Myosins are contractile proteins that are highly expressed in myoepithelial cells (MECs) and present around salivary gland ducts and acini to help maintain normal salivary flow. The aim of this study is to investigate the effect of BTX injections on the submandibular salivary glands of adult female Albino rats, when administered solely or in conjunction with Epidermal growth factor (EGF) through immunohistochemical localization of myosin in the parenchyma of the gland. Sixty rats were used in this study and were equally divided into control (saline) group, BTX group and EGF + BTX group (Combined treatment). The results obtained from this study showed that myosin expression in submandibular salivary glands of rats significantly decreased after a single subcutaneous injection of 2.5 units of BTX in 0.1ml saline. However, daily intraperitoneal injections of EGF with a dose of 10 µg/Kg body weight restored normal levels of myosin expression, as well as normal integrity and function of submandibular salivary glands. Further confirmation of the above findings is recommended through immunohistochemical localization of E-cadherin as well as ultrastructural examination of submandibular salivary glands treated with BTX and EGF.

Key words: Submandibular salivary gland, Epidermal growth factor, Botulinum toxin, Clostridium, Myosin.

Corresponding Author: Dr. Mahmoud M Bakr, Lecturer, General Dental Practice, School of Dentistry and Oral Health, Griffith University, Queensland, 4222, Australia

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INTRODUCTION:

Nowadays, patients are demanding not only enhancement to their dental (micro) esthetics, but also their overall facial (macro) esthetics. Over the past decade, facial rejuvenation procedures to circumvent traditional surgery have become increasingly popular. Office-based, minimally invasive procedures can promote a youthful appearance with minimal downtime and low risk of complications. Injectable BTX, soft-tissue fillers, and chemical peels are among the most popular non-invasive rejuvenation procedures, and each has unique applications for improving facial aesthetics. Soft tissue augmentation via dermal filling agents may be used to correct facial defects such as wrinkles caused by age, gravity, and trauma; thin lips; asymmetrical facial appearances; buccal fold depressions; and others. [1]

BTX, popularly known by its trade name botox, is a protein and neurotoxin produced by the bacterium Clostridium botulinum. It is the most potent poison known, which has often been feared as a possible biological weapon.[2] In 1817, Justinus Kerner, a German poet and physician, described BTX as a "sausage poison" and "fatty poison", because the bacterium that produces this toxin often caused poisoning by growing in improperly handled or prepared meat products. The author provided the first account of food borne botulism. From this outbreak, he recognized the potential of the toxin as a therapeutic agent. He noticed that the toxin paralysed skeletal muscles and parasympathetic function. Kerner hypothesized that the toxic substance causing botulism could be helpful in treating hypersalivation, as severe dry mouth was one of the first manifestations of botulism. [3]

The BTXs work by inhibiting the release of the neurotransmitter, acetylcholine, at the neuromuscular junction thus causing muscle relaxation. Acetylcholine is also the neurotransmitter in postganglionic fibres of the parasympathetic division of the autonomic nervous system. These fibres innervate various glands, such as the salivary glands. [4] By inhibiting the release of acetylcholine at the neuroglandular junction, a temporary salivary flow rate reduction can be achieved. [5, 6]

The cells in our body constantly communicate with each other, negotiating the transport and use of resources and deciding when to grow, when to rest, and when to die. Often, these messages are carried by small proteins, such as epidermal growth factor (EGF). EGF is a message, telling cells that they have permission to grow. It is released by cells in areas of active growth, and then is either picked up by the cell itself or by neighbouring cells, stimulating their ability to divide. The message is received by a receptor on the cell surface, which binds to EGF and relays the message to signalling proteins inside the cell, ultimately mobilizing the processes needed for growth. [7]

Botulinum toxin may serve as a valuable addition to the beneficial non-cosmetic treatment a dentist can provide to his or her patient. Although other traditional solutions are available, research shows that BTX is a viable treatment for many facial and oral musculature dysfunctions. BTX provides an overall conservative, quick and painless approach. Patients must no longer endure surgeries, take medications, or, in some cases, participate in behavioural modifications to relieve their muscle pains and/or maintain an appropriate quality of life. [8]

BTX can be used in the following dental disorders:

- Temporomandibular disorder (TMD).
- Dental implants and surgery.
- Prominent gums (Gummy smile).
- Masticatory Muscles hypertrophy.
- Myofacial pain and neck pain.
- Salivary glands disorders.

In many exocrine glands, like the salivary glands, the secretory end pieces and the ducts are partly covered by cells with long processes that form an interlacing network. These cells resemble smooth muscle cells in several important aspects, yet clearly are epithelial cells, and thus are referred to as myoepithelial cells (MEC). Myoepithelial cells are important for maintaining salivary flow and the integrity of salivary glands.[9] In the present study the expression of myosin in myoepithelial cells as well as other parenchymal and stromal elements of the rat submandibular salivary glands after treatment with BTX and epidermal growth factor is investigated.

AIM OF THE STUDY:

The aim of the present investigation is to study the possible effects of the most commonly used anti-wrinkle agent Botulinum toxin (Botox) either separately or coupled with epidermal growth factor on the submandibular salivary glands of adult female Albino rats through immunohistochemical localization of myosin in the parenchyma of the gland.

MATERIALS AND METHODS:

Sixty healthy adult female Albino rats, three months old and 200-220 gm in body weight were used in this study. The rats were obtained from Kasr el Aini animal experimental unit, Faculty of Medicine, Cairo University. The rats were housed in separate cages, five rats per cage and kept in an environment with controlled temperature (25°C), humidity (45%-75%), and photoperiod (12:12 hour light-dark cycle). The animals were fed natural diet and supplied drinking water ad libitum throughout the whole experimental period. The rats were acclimatized for one week before the initiation of the experiment.

The animals were randomly divided into three groups as follows:

Control group: consisted of 20 rats, subjected to subcutaneous injection of 0.1 ml saline in the region of the right and left submandibular salivary glands and served as controls.

Botox (BTX) group: consisted of 20 rats that were subjected to a single dose of subcutaneous injection of 2.5 unit botulinum toxin type A (Botox 100 units Allergen), reconstituted in 0.1 ml of physiologic saline in the region of the right and left submandibular salivary glands. [10]

EGF group (Botox + Epidermal growth factor): consisted of 20 rats, that were injected once subcutaneously with 2.5 unit botulinum toxin in 0.1 ml saline as in BTX group and on the next day they were subjected to daily intra peritoneal injection of epidermal growth factor, provided by Sigma-Aldrich, Inc. in a dose of 10 µg/Kg body weight [11] for sixty days.

At the end of the experiment, all animals were sacrificed by euthanization, their right and left submandibular salivary glands were dissected out. Submandibular salivary glands were processed for histological examination and stained with Immuno-peroxidase staining for immunohistochemical localization of myosin in the glandular tissue using staining reaction incubated by anti-myosin.

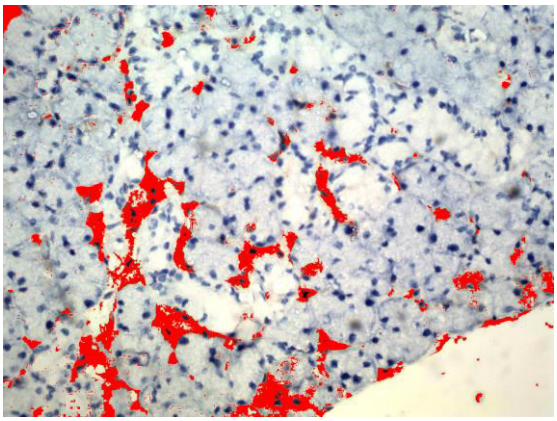
For myosin immunohistochemical localization, negative controls were prepared by substituting the primary antibody by a nonspecific serum of the same dilution as its respective antibody and the procedures were continued as usual. Six sections from each submandibular salivary gland stained with antimyosin antibody were selected, examined with ZEISS Primo Star light microscopy and photographed by Tucsen IS 1000 10.0MP Camera in the Oral Biology lab, Faculty of Dentistry, British University in Egypt**.**

The staining reaction of the different immunohistochemical parameters of the different groups was scored as follows:

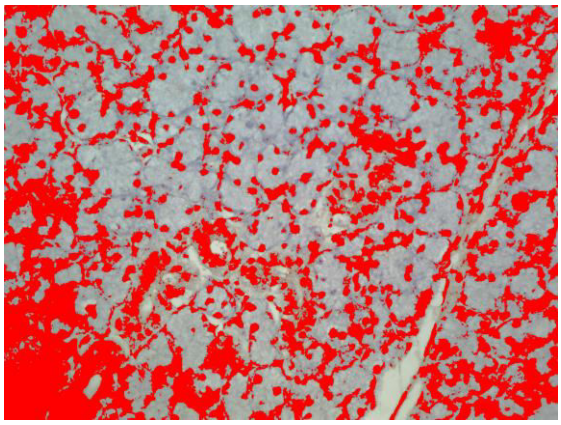
(-) negative staining reactivity.
(+) weakly positive staining reactivity.
(++) moderately positive staining reactivity.
(+++) strongly positive staining reactivity.

The intensity of the immunohistochemical staining results from different groups were histometrically analyzed using Image J (1.46 a, NIH, USA) computer system (1.46 a, NIH, USA). For each selected section, six microscopic fields were selected and captured at a magnification 200X using a digital video camera mounted on a light microscope (CX21, Olympus, Japan). Images were then transferred to the computer system for analysis.

Images were manually corrected for brightness and contrast. Color thresholding was then performed automatically after which pictures were converted to RGB stack type. Masking of the brown myosin, immuno-stain was performed by red color where any brown stain of any intensity was considered positive whereas the background grey stain was considered negative (Fig 1). Area fraction was then calculated automatically representing the area percentage of immune positive cells to the total area of the microscopic field.



(a)



(b)

Figure 1: Showing an example of myosin expression in the BTX group (a) and in the EGF group (b). Myosin expression was masked in red color using the Image J software.

All data obtained from histomorphometric analysis was statistically described in terms of range, mean, ± standard deviation (±SD), and median. Comparison between groups was done using Kruskal Wallis analysis of variance (ANOVA) test with Conover-Inman test for independent samples as post hoc multiple 2-group comparisons. A p value less than 0.05 was considered statistically significant. All statistical analysis was done using Microsoft Excel 2013 (Microsoft Corporation, NY, USA) and Statistical Package for the Social Sciences version 21 (SPSS Inc., Chicago, IL).

RESULTS:

Negative control:

Sections taken from the submandibular salivary glands of rats from the control group and incubated with non-specific serum and colour developed by AEC revealed negative staining reaction of all elements forming the glands (Fig. 2).

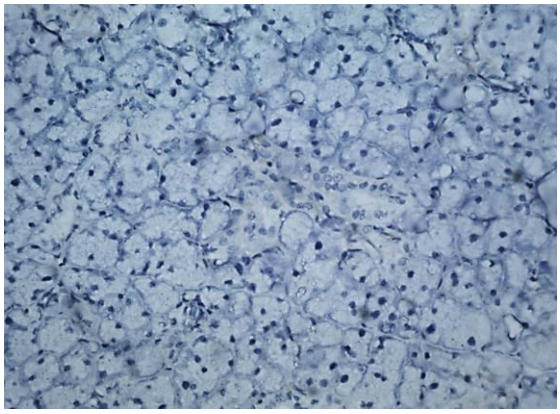


Figure 2: A photomicrograph of rat submandibular salivary gland incubated with non-specific serum and colour developed by AEC showing negative staining reaction of all the gland components (AEC original mag. x 100).

Control group:

Weakly positive staining of the serous acini cytoplasm was evident, while that of mucous acini was negative. Also, weakly positive staining was observed in the intercalated, striated and excretory ducts as well as granular convulated tubules. Strongly positive staining reaction was found around most of the secretory acini and intercalated ducts representing staining of the myoepithelial cells with their elongated cell bodies and extensions of their processes (Fig 3).

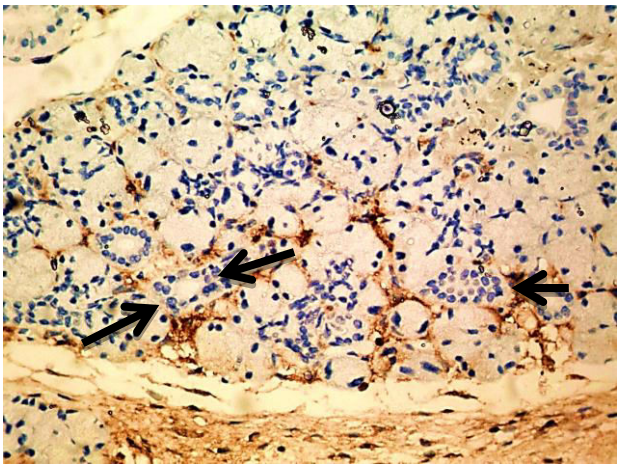


Figure 3: A photomicrograph of the rat submandibular salivary gland of control group showing weakly positive staining of the serous acini and strongly positive staining of the myoepithelial cells to myosin (arrow) (antimyosin original mag. x 100).

Botox (BTX) group:

Negative to weakly positive staining of the serous acini cytoplasm was evident, while that of mucous acini was negative. Weakly positive staining reaction around most of the secretory acini and intercalated ducts representing weak staining of the myoepithelial cells with their elongated cell bodies and extensions of their processes. Weakly positive staining of intercalated, striated and excretory ducts as well as granular convulated tubules was also evident (Figs 4 & 5).

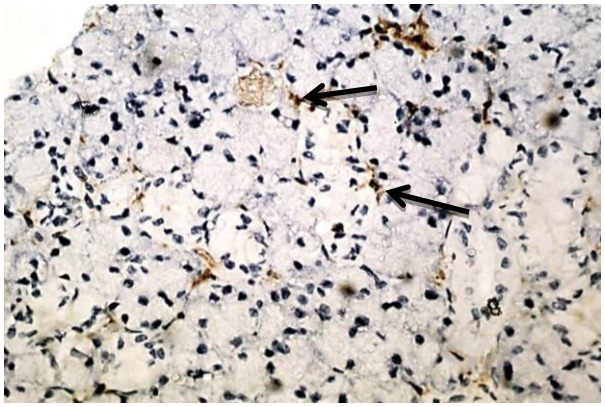


Figure 4: A photomicrograph of the rat submandibular salivary gland of control group showing negative to weakly positive staining of the serous acini and weakly positve staining of the myoepithelial cells to myosin (arrow) (antimyosin original mag. x 200).

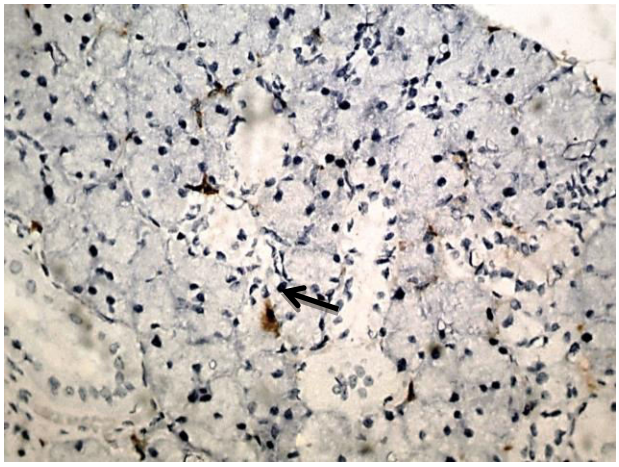


Figure 5: A photomicrograph of the rat submandibular salivary gland of control group showing negative to weakly positive staining of the serous acini and weakly positive staining of the myoepithelial cells to myosin (arrow) (antimyosin original mag. x 200).

EGF group:

Negative to weakly staining of the cytoplasm of serous secretory cells. While mucous acini showed negative staining. Strongly positive reaction around most of the secretory acini and intercalated ducts representing staining of the myoepithelial cells with their elongated cell bodies and extensions of their processes. The intercalated ducts, striated ducts, granular convoluted tubules, and excretory ducts revealed weakly positive staining reaction (Fig 6).

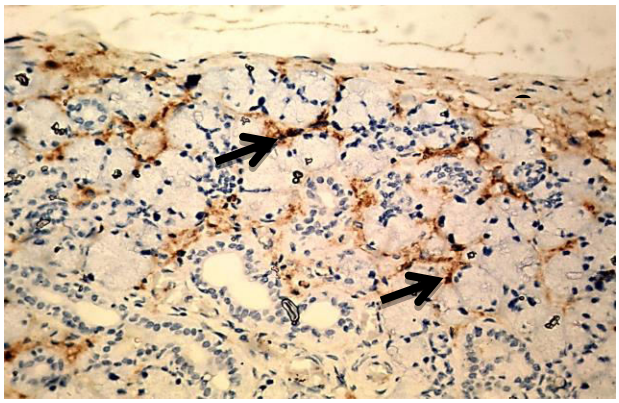


Figure (6): A photomicrograph of the rat submandibular salivary gland of EGF group showing strongly positive staining of the myoepithelial cells to myosin (arrow) (antimyosin original mag. x 100).

Table 1: Illustrates the staining intensity scores of the submandibular salivary gland components to myosin.

Group	Control	Botox	EGF
Serous cells	+	-/+	-/+
Mucous cells	-	-	-
Myoepithelial cells	+++	+	+++
Intercalated ducts	+	+	+
Striated cells	+	+	+
Granular convoluted tubules	+	+	+
Excretory ducts	+	+	+
Connective tissue cells	+	+	+
Wall of blood vessels	+	+	+

- Negative staining reactivity.
+ Weakly positive staining reactivity.
++ Moderately positive staining reactivity.
+++ Strongly positive staining reactivity.

STATISTICAL RESULTS:

Histomorphometric analysis of the three groups of the submandibular salivary gland revealed that the highest mean area percentage occupied by myosin immunostaining was recorded in the control group, whereas the lowest value was recorded in the BTX group.

Statistical analysis of variance (ANOVA test), revealed that the difference in the mean area percentage of expression of myosin immunostaining was extremely statistically significant ($p<0.0001$). The mean values of the expression of mysoin area percentage in all groups of submandibular salivary gland are summarized in table (2) (Figs. 7 & 8).

Table 2: Illustrates the mean values and standard deviations (SD) of the area percentage of myosin expression in the submandibular salivary gland of all groups.

Group	Control group	BTX group	EGF group
No. of cases	20	20	20
Max	38.97	16.21	31.75
Min	30.68	12.96	21.63
Mean	34.72	14.01	27.67
SD	2.95	1.38	2.84
Median	35.00	14.66	27.40

Comparison between different groups:

A pair wise comparison was held between myosin mean area expression percentage in control group and the other experimental groups using an unpaired (independent) Student's t-test (Table 3).

There was an extremely statistically significant decrease in the myosin expression area percentage in the BTX group when compared to the control group ($p<0.0001$). While there was a slight decrease in the myosin expression area percentage in the EGF group when compared to the control group, where this decrease was statistically insignificant. By comparing myosin expression area percentage between BTX group and the EGF group, there was an extremely significant increase in myosin expression area percentage in the EGF group when compared to the control group ($p<0.0001$) (Table 3).

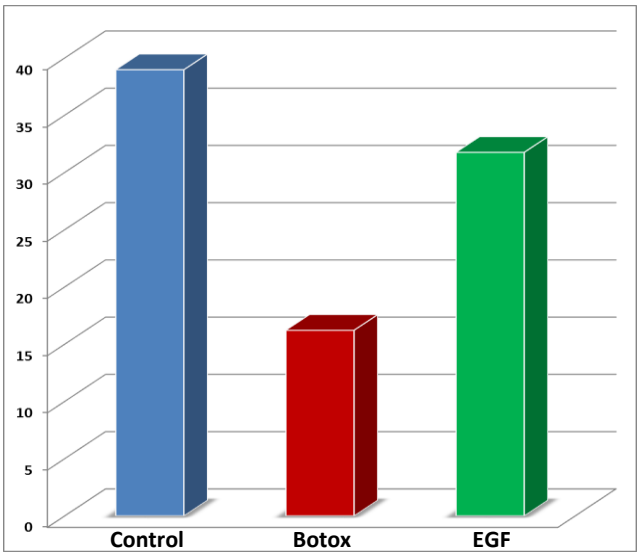


Figure 7: Bar chart showing the myosin mean area percentage expression in submandibular gland between all the groups.

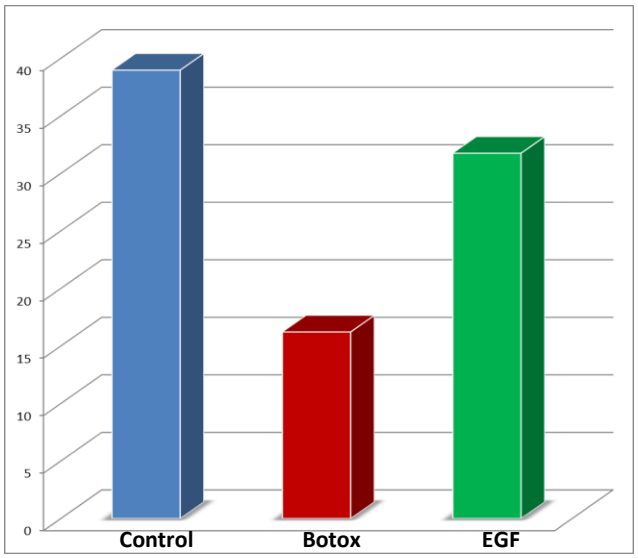


Figure 8: Bar chart showing the myosin maximum mean area percentage expression in submandibular gland between all the groups.

Table 3: Illustrates the results of the descriptive statistical pairwise comparison between area percentage of myosin expression in the submandibular salivary gland of all groups.

Comparison	Mean difference L	T value	P value
Control vs. Botox	20.7178	25.21454	< 0.0001***
Control vs. EGF	7.0554	11.23165	0.4545**
Botox vs. EGF	13.6645	18.45545	< 0.0001***

*** extremely statistically significant
** insignificant

DISCUSSION:

BTX Type A (BTXA) was used in this study as it has a greater anticholinergic effect at the neuromuscular junction than any other type of BTX and only type A and most recently B are approved for clinical use.[12] Female Albino rats were used in the present study because according to the American Society of Plastic Surgeons, 91% of all cosmetic procedures are performed on females. Myosin expression was investigated to detect the myoepothelial cells (MEC) which are epithelial cells yet resembling smooth muscle cells which contain actin and myosin filaments necessary for their contraction[13]

In the current study, myosin was weakly expressed in the BTX treated group in contrast to the control group where there was a strong expression around the periphery of serous acini as wells as the intercalated ducts monitoring the myoepithelial cells (MEC). This difference in the reaction pattern denotes a decrease in the intracellular content of myosin in BTX injected rats which is one of the markers that readily identifies MEC in salivary glands. The decrease in the myosin content might explain the change in the shape of the acini and ducts. It was reported that any decrease in myosin and actin contractile proteins is associated with change in the characteristic shapes of the cells.[14] Moreover, the defect in the myosin content of the MEC results in impairment of its contractile function and may result in defective excretory function of acini and ductal system and subsequently leading to stagnant salivary secretion and possibly lowering the rate of salivary flow. In this study, myosin expression was nearly similar in both EGF and control groups where a strongly positive reaction was found around the acini and intercalated indicating normal structure of myoepithelial cell which is epithelial in origin. This may be due to the fact that EGF is involved in regulating cellular proliferation, differentiation, and survival and EGF also facilitates epidermal cell regeneration.

CONCLUSION:

This study demonstrated that BTXA injection lead to a deleterious effects on the submandibular salivary gland tissue while EGF treatment improves the condition. This was confirmed through immunohistochemical localization of myosin.

RECOMMENDATIONS AND FUTURE RESEARCH:

Further research is being conducted by our team to investigate the effect of EGF on the submandibular salivary glands in botox treated albino rats through localization of E-cadherin. The findings obtained from all immunohistochemical markers will be confirmed through histological and ultrastructural examination.

ACKNOWLEDGEMENTS:

This project was reviewed and granted ethical approval by the Suez Canal University Research Ethics Committee (SUEZ-REC 35/2014).

REFERENCES:

1. Dastoor SF, Misch CE, Wang HL. Dermal fillers for facial soft tissue augmentation. J Oral Implantol. 2007; 33(4):191-204.
2. Arnon SS, Schechter R, Inglesby TV, Henderson DA. Botulinum toxin as a biological weapon: medical and public health management. JAMA. 2001; 285(8): 1059-1070.
3. Erbguth FJ. From poison to remedy: the chequered history of botulinum toxin. J Neural Transm. 2008; 115(4):559-565.
4. Aoki KR, Smith LA, Atassi MZ. Mode of action of botulinum neurotoxins: current vaccination strategies and molecular immune recognition. Crit Rev Immunol. 2010; 30(2):167-87.
5. Jongerius PH, Rotteveel JJ, van Limbeek J, Gabreëls FJ. Botulinum toxin effect on salivary flow rate in children with cerebral palsy. Neurology. 2004; 63(8):1371-1375.
6. Shan XF, Xu H, Cai ZG, Wu LL, Yu GY. Botulinum toxin A inhibits salivary secretion of rabbit submandibular gland. Int J Oral Sci. 2013; 5(4):217-223.
7. Zeng F, Harris RC. Epidermal growth factor, from gene organization to bedside. Semin Cell Dev Biol. 2014; 28:2-11.
8. Hoque A, McAndrew M. Use of botulinum toxin in dentistry. NY State Dent J. 2009; 75(6):52-55.
9. Chitturi RT, Veeravarmal V, Nirmal RM, Reddy BV. Myoepithelial Cells (MEC) of the Salivary Glands in Health and Tumours. J Clin Diagn Res. 2015; 9(3):14-18.
10. Ellies M, Gottstein U, Rohrbach-Volland S, Arglebe C, Laskawi R. Reduction of salivary flow with botulinum toxin: extended report on 33 patients with drooling, salivary fistulas, and sialadenitis. Laryngoscope. 2004; 114(10):1856-1860.
11. Kane CD, Nuss JE, Bavari S. Novel therapeutic uses and formulations of botulinum neurotoxins: a patent review (2012 - 2014). Expert Opin Ther Pat. 2015; 25(6):675-90.
12. Small R. Botulinum toxin injection for facial wrinkles. Am Fam Physician. 2014; 90:168-75.
13. Sellers JR. Myosins: a diverse superfamily. Biochim Biophys Acta. 2000; 1496(1):3-22.
14. Cooper GM. The cell: A molecular approach; Actin, Myosin, and cell movement. 2nd edition, Sunderland (MA): Sinauer Associates. 2000.

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